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# Fast Genetic Variation among Coliphage Quasispecies Revealed by a Random Amplified Polymorphic DNA (RAPD) Analysis

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**Genetic analysis was conducted on newly isolated coliphages from soil by using a RAPD assay. From the initial result, the coliphages were turned out to be different from one another but were closely related to φλ due to the fact that they shared the same RAPD marker in which other T phage testings failed to show. By using the primers EC01 or EC02, a fast genetic mutation of φC1 was found by producing specific RAPD markers on the phages from the first filial progeny to the second filial progeny. When we made a RAPD assay with combined primers (EC01, EC05 and EC 08), the genetic mutation was again confirmed in φC1. The assay detection showed mutations in other coliphages such as φC2 and φC3 by revealing specific RAPD bands among different progeny phages, where genetic instability of the coliphages is implied.**

**Key Words: Random amplified polymorphic DNA (RAPD), coliphages, genetic variation, quasispecies**

Coliphages are bacterial viruses harbored in *Escherichia coli*. And they play an important role in this complex and diverse environment for its microbial population. Especially soil coliphages are great in number so that they provide very good sources for studying genetic diversity (15). And that is proved by many other researchers who continuously isolate new coliphages (4,5).

The random amplified polymorphic DNA (RAPD) analysis was originally devised by Williams *et al.* (13) and Welsh and McClelland (11) as an *in vitro* DNA amplification technique. It employs short synthetic oligonucleotides as a random primer to exponentially increase the template DNA by a polymerase chain reaction. The technique is known to be a very useful means of analyzing genetic structures and relationships in plants (13), fungi (16), bacterial species (2,7), animals (6) and viruses (5). It is now widely used in the area of studying genetic mapping, diagnostics, and taxonomy (12).

This study was designed to find genetic relationships among the isolated coliphages as a continuing study for characterization of the coliphages isolated from soil. Since genetic variation of the species usually starts from a minor nucleotide change and its accumulation, we applied the RAPD technique which provides highly sensitive detection in distinguishing nucleotide difference (14). In fact, such a mutation would cause a quasispecies in the population of coliphages that is derived from the continuous emergence of mutant viruses during replication. Thus, we also investigated genomes of the successively propagating progeny of the coliphages by the RAPD assay in order to understand the degree of variation which occur in the coliphages. That is to understand, the characteristics of genetic variation which occur among many animal viruses and which give dynamic equilibrium for genetic diversity in many types of viruses.

## Materials and Methods

### Preparation of coliphages and their progenies

Coliphages were isolated from wet soil of paddy fields near Keimyung University and their host used was *Escherichia coli* C1 (KCTC #2282). After the isolation of coliphages φC1, φC2, φC3 and φC4, the coliphages were purified and characterized as mentioned in Kwon (5). Progeny phages (1st filiation to 4th filiation) were obtained by repeating plaque assays described in Sinsheimer (10). Medium used was Luria broth (LB) which was

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made from polypeptone 10 g, yeast extract 5 g, NaCl 5 g, and glucose 1 g in 1,000 ml of distilled water. When necessary, agarose was added to LB for making 1.5% agar plates.

### Coliphage DNA extraction

Phage particles were collected in TEM (10 mM Tris-HCl, pH7.6; 1 mM EDTA; 10 mM $MgCl_2$). After two runs of differential centrifugation (2,500 rpm with Vision 6000CF and 12,000 rpm with Vision VS15000), a ml of supernatant solution was mixed with 10 μl RNase(10 mg/ml) and 2.5 μl DNase I (5 mg/ml). After incubation at 37°C for 3 hours, the mixture was added with 10 μl of proteinase K (20 mg/ml) and incubated at 37°C overnight. Subsequently, the mixture was treated twice with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and DNA was collected after ethanol precipitation. RNase was treated once more to the DNA in order to make sure the removal of the bacterial ribosomal RNA. After phenol treatment and ethanol precipitation, the DNA was analyzed by electrophoresis to measure the size of coliphage genomes (data not shown). For a RAPD assay, all phage DNA were purified once again by electrophoresis using 0.7% agrose gel. Then, 10 or 20 μl of DNA solution was applied directly to the PCR reaction. The control phage (φT2, φT4, φT5, φT7, and φλ) DNAs used in this experiment were purchased from Sigma Chemical Co.

### Random primers

Random primers used in this experiment were obtained from Dr. John Hobbs of University of British Colombia, Vancouver. Among them, oligonucleotides (10 mer) having GC contents of 50~80% were applied for the purpose of phage genome amplification. The nucleotide sequences of random primers which gave successful results throughout this study, are listed in Table 1.

**Table 1.** Nucleotide sequences of random primers used in this

| Primers | Sequences | G/C% |
|---|---|---|
| EC01 | 5'-GCGGCTGGAG-3' | 80 |
| EC02 | 5'-GCGGTTGAGG-3' | 70 |
| EC03 | 5'-AGCAGCGTGG-3' | 70 |
| EC04 | 5'-GAAGGCTCTG-3' | 60 |
| EC05 | 5'-TGTCGGTTGC-3' | 60 |
| EC06 | 5'-GTATTGCCCT-3' | 50 |
| EC07 | 5'-TGTACGTGAC-3' | 50 |
| EC08 | 5'-GCTTGTGAAC-3' | 50 |
| EC09 | 5'-CTTTCGTGCT-3' | 50 |

### RAPD assay using PCR

Polymerase chain reaction (PCR) was carried out to amplify the coliphage genomic DNA with many random primers. The reaction condition was as follows: Each 10 μl of purified phage DNA was added to 40 μl of PCR reaction mixture, containing 10X *Taq* buffer, 40 mM dNTPs, oligonucleotide primers (200 pmol each), and 1 unit of *Taq* DNA polymerase (Perkin-Elmer). The 10X Taq buffer contained 500 mM Tris-HCl (pH 8.3), 25 mM $MgCl_2$ and 0.1% gelatin. Two drops of mineral oil were added to each reaction tube. In an automatic thermocycler (Appligene, UK), the reaction tubes were subjected to following temperature cycles: 95°C for one minute, 40°C for one minute and 72°C for two minutes. After 42 cycles of amplification, the samples were incubated for 10 minutes at 72°C.

### Agarose gel electrophoresis and UV photography

One fifth of the PCR reaction (10 μl) was loaded on a gel containing 2% NuSieve GTG agarose (FMC) in Tris-Borate buffer for electrophoresis. For convenience, 0.5 μl of ethidium bromide solution (10 mg/ml) was directly added to each 10 ml agarose solution for DNA staining. After running the gel, it was subjected to UV photography as described by Sambrook *et al.* (9) in order to verify the PCR reaction results.

## Results and Discussion

### Some aspects of a RAPD assay for coliphages

Since coliphages are one of the simplest organisms and they have very small size genomes compared with larger and more complex genomes of higher organisms, we expected that RAPD results of coliphages would be different from other RAPD works. For instance, we thought that the genomic DNA of coliphages would not yield as many RAPD bands as RAPD bands found in the samples of most eukaryote genomes because the size of the coliphage genomes was extremely small ($3\times10^4$ bp vs. $3\times10^9$ bp), leading to lessening the chance for binding random primers.

In fact, we did fail to produce RAPD bands from the genomes of coliphages with most of the random primers tested but the nucleotide sequences of the few random primers which were suitable to amplify the coliphage DNA, are listed in Table 1. Typical RAPD banding patterns for the coliphages are presented in Fig. 1. A very strong RAPD band was found with the DNA size of 603 base pairs. The band appeared in all coliphage DNA samples (lanes 1 to 4) as a coliphage marker whereas all T phages failed to produce the 603 bp band (lanes 5 to 8). However, φλ (lane 9) showed the same 603 bp band which would im-

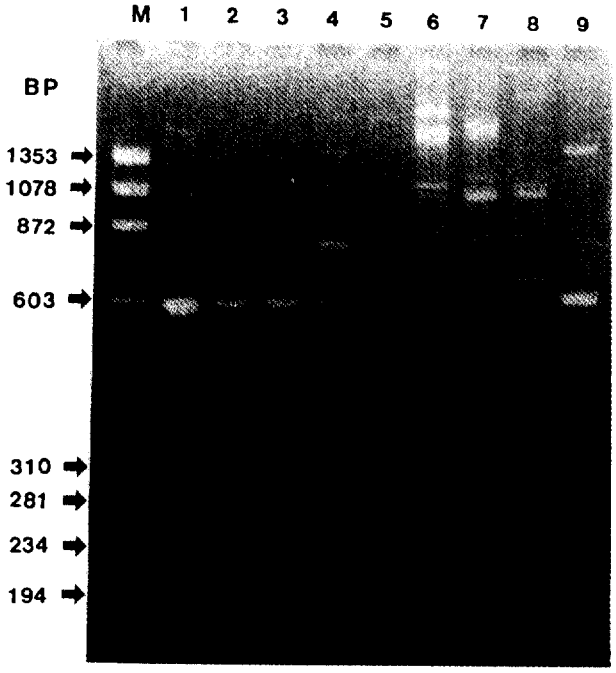


**Fig. 1.** Typical random amplified polymorphic DNA banding patterns of various coliphages. A RAPD assay was done with two kinds of random primers, EC02 and EC09. PCR was carried out with 40 thermocycles at 95℃ denaturation for 1 minute, 40℃ annealing for 1 minute and 72℃ polymerization for 2 minutes. Coliphage DNAs used were: ϕC1, ϕC2, ϕC3, ϕC4, ϕT2, ϕT4, ϕT5, ϕT7 and ϕλ (lanes 1 to 9, respectively). M is a ϕX174 *Hae*III digested DNA (0.2 μg).

ply that the islated coliphages were more closely related to ϕλ than the T phages. The coliphages and T phages were also distinguished by the difference in the RAPD patterns presented in Fig 1. The RAPD band patterns of T phages used as controls (lanes 5 to 8) were very different from those of coliphages (lanes 1 to 4). With the same primers (EC02 and EC09), the genomes of T phages showed many high molecular weight DNA bands whereas all four coliphages showed relatively smaller RAPD bands. Then, we concluded that the RAPD analysis could be used to identify the nature of coliphages at the level of their genomic DNAs that were different between coliphages and T phages.

We also examined an effect of GC contents of the random primers that may be a critical parameter for ensuring successful RAPD assays throughout this study. Since the GC contents of primers is one of the key factors affecting primer melting temperature (Tm), they will exert upon primer binding to the templates during polymerase chain reaction (8). We tested the primers having GC contents from 60% (EC04, Tm : 32℃) to 80% (EC01, Tm : 36℃) with the ϕC1 DNA that was extracted from different progeny (first filiation to 4th filiation). The results are shown in Fig. 2. With the primer EC01, it revealed a more specific marker that was detected only from the first filial progeny of C1 (lane 1) while other successive filial progeny did not produce the band just above 603 bp DNA (arrow). A primer EC 02 also produced a specific RAPD marker (arrow) near 310 bp DNA, which was found in the F2 to F4 but not in the F1 progeny. In other words, the DNA sequence of the first filial progeny of ϕC1 was not the same as other successive filial progeny (F2 to F4). There were nucleotide changes from the genome of F2 progeny. This evidence tells us that DNA sequence of the first filial progeny of ϕC1 was different from that of other successive filial progenies of ϕC1. Thus, we can refer them as 'quasispecies' of coliphage ϕC1 because they differed in their nucleotide sequences from the wild type of ϕC1 (1). The 2nd filial coliphage of the ϕC1 was a molecular variant that consisted of the ϕC1 quasispecies.

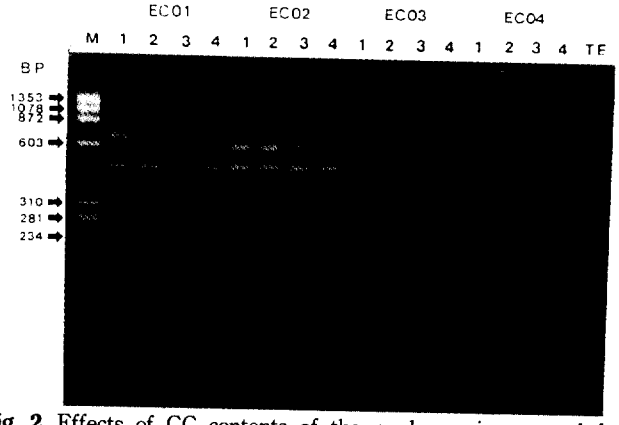


**Fig. 2.** Effects of GC contents of the random primers used for the RAPD analysis. The GC contents of used primers were 80% (EC01), 70% (EC02), 70% (EC03), 60% (EC04). The random primer sequences are shown in Table 1. M is a DNA marker (0.2 μg of ϕX174 *Hae*III digested) and TE is referred to as $T_{10}E_1$ buffer which was used as a PCR negative control. 1 (1st filial progeny); 2 (2nd filial progeny); 3 (3rd filial progeny); 4 (4th filial progeny). Sharp arrows(→) indicate location of specific RAPD markers for primers EC01 and EC02. Sample DNA used was coliphage ϕC1. PCR conditions were mentioned in Materials and Methods.

## RAPD markers revealing conserved sequence of the isolated coliphages

Since quasispecies of the ϕC1 also produced common RAPD markers as shown in Fig. 2 (EC03), a test was developed to verify the existence of coliphage quasispecies with a combined set of random primers that is used in combination of three different kinds of primers such as primers EC01, EC06 and EC07. We expected that there would be more RAPD bands gen-

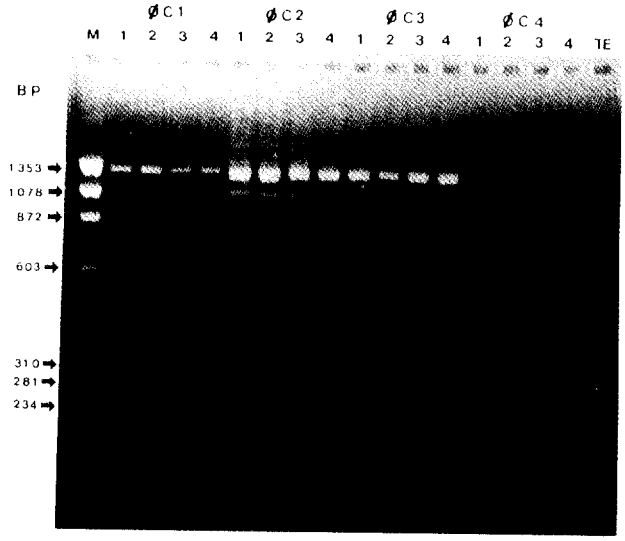


**Fig. 3.** Mostly conserved DNA bands through the coliphages with random primers EC01, EC06 and EC07. Lane descriptions are M (0.5 μg of φX174 *Hae*III digested); 1 (1st filial progeny); 2 (2nd filial progeny); 3 (3rd filial progeny); 4 (4th filial progeny) and TE ($T_{10}E_1$ buffer) as a negative control. From left to right side of the photograph, coliphage DNAs were φC1, φC2, φC3 and φC4. PCR conditions were mentioned in Materials and Methods.

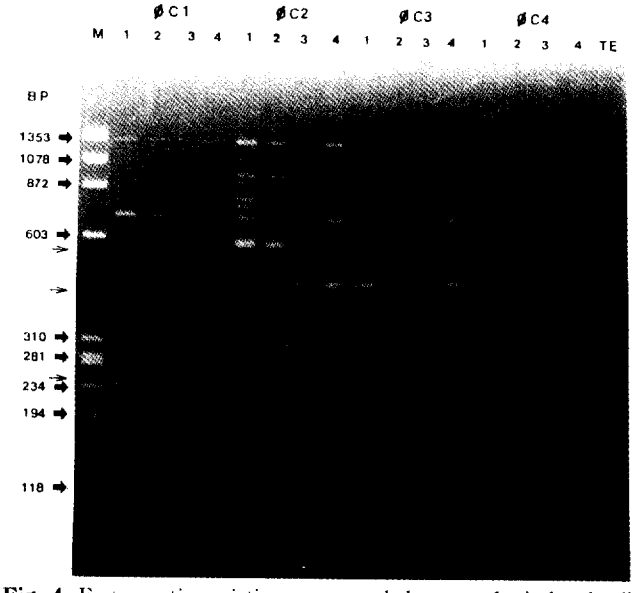


**Fig. 4.** Fast genetic variation was revealed among the isolated coliphages and the coliphage progeny by the RAPD assay. Some specific RAPD markers of the progeny phages were marked with sharp arrows (➡). Used primers were EC01, EC05 and EC08. From left to right side of the photograph, coliphage DNAs were φC1, φC2, φC3 and φC4. Lane descriptions are M (0.2 μg of φX174 *Hae*III digested); 1 (1st filial progeny); 2 (2nd filial progeny); 3 (3rd filial progeny); 4 (4th filial progeny) and TE ($T_{10}E_1$ buffer) as a negative control.

erated after PCR because using the combined primers can amplify a wide variety of genome segment than using a single primer (3). Therefore, it can be resolved more precisely when compared with a single primer. Unexpectedly, this test revealed several strong common RAPD bands that were produced throughout the successive filial progeny, from φC1 to φC4 (Fig. 3). The DNA bands were somewhat high molecular weight DNA bands (e.g., 1,353 bp and 1,078 bp). It was interesting that they appeared throughout all coliphage samples, and we reasoned that the high molecular weight DNA bands were generated from the most conserved DNA sequences of the coliphages. Probably, the DNA bands are related to nucleotide sequences of some phage specific proteins that must be conserved throughout coliphage replication, i.e., polymerases or lytic enzymes that are necessary for fast phage replication and plaque formation. From this result, we can deduce two facts: 1) these strong RAPD markers represent typical RAPD markers of the isolated coliphages which infect mainly *E. coli* C1, 2) among the quasispecies of the coliphages, certain DNA sequences must be preserved for their existence although we found a fast genetic variation occurs in the φC1 progeny. To understand these ideas, a further study is now being carried out at a molecular level.

## Genetic variation among coliphage quasispecies

Although all the isolated coliphages had DNA as their genomes, a fast nucleotide change among φC1 quasispecies was detected by a RAPD assay as shown in Fig. 2. Then the same logic was tested to reveal the genetic variations among the other coliphages and quasispecies with different random primers which would generate different RAPD markers. When a primer set such as EC01, EC05 and EC08 was applied to the samples, surprisingly, a very different RAPD pattern had vividly appeared. It implied that a strong genetic variation had occurred within a relatively very short interval. This variation from generation to generation was shown in Fig. 4.

The φC1 samples again showed the same result we found in the Fig. 2. That is, the first filiation of φC1 had a specific RAPD marker (sharp arrow near 234 bp DNA) that was not found from the 2nd, 3rd, 4th filiation progeny of φC1. This confirmed that there was genetic mutation from F1 to F2. φC2 also revealed one specific RAPD band at the third filiation sample between 603 bp and 310 bp DNA band (lower arrow) while the 2nd filiation of φC2 had a specific RAPD band near 603 bp DNA (upper arrow). In the case of φC4, it was hard to find specific RAPD markers with the combined primer set but genetic mutation among the successive filiation

**Fig. 5.** Plaque morphology of the coliphages ϕC2 and ϕC3, which showed very similar RAPD markers in Fig. 3 and Fig. 4. ϕC2 (panel A) made medium size (4~5 mm) plaques with round margin and ϕC3 (panel B) made small size (2~3 mm) plaques with rough margin.

was detected by using primer EC02 (data not published).

Thus, some specific RAPD bands found in Fig. 4 simply tell us that dynamic nucleotide mutations were occurring from generation to generation among the isolated coliphages. Although this genetic variation was made in a very short time, it was easily detected by a sensitive method like the RAPD assay. So far, this kind of variation among the quasispecies of coliphages seemed to be insignificant to the coliphage population. However, we now understand that the fast and dynamic mutations could be constantly occurring among the coliphages with DNA genomes. It may eventually cause the accumulation of mutant coliphages that would affect the *E. coli* population dominated in soil.

As a proof, it was found that the RAPD markers of ϕC2 and ϕC3 were very similar when we closely examined them in Fig. 4. The fact that the two coliphages were originally isolated from the same soil sample and other supporting data (5) confirmed their closeness on a genetic level. Interestingly enough, their plaque morphology was not the same (Fig. 5). Plaques of ϕC2 (plate A) were bigger with round margin compared with those of ϕC3 (plate B) which had small rough margined plaques. This discrepancy tells us that they were mutants derived from a same quasispecies but their genetic variations were fixed as their phenotypes which were demonstrated in their plaque morphology.

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## References

1. **Cann, A.J.,** 1994. Principles of molecular virology. p. 52-53. Academic Press, London.
2. **Cho, M.J., W.K. Lee, Y.S. Jeon, K.H. Kim, S.H. Kim, S. C. Baik, K. H. Rhee, Y.O. Kim, H.S. Yoon, and N.S. Kim,** 1995. Intrafamilial transmission of *Helicobacter pylori* detected by random amplified polymorphic DNA fingerprinting. *Mol. Cells.* **5**, 508-513.
3. **Dear, P.H. and P.R. Cook,** 1989. Happy mapping: A proposal for linkage mapping the human genome. *Nucleic Acids Res.* **17**, 6795-6807.
4. **Hong, S.H., N.Y. Cho, and G. Jeong,** 1994. Isolation and partial characterization of a new *Escherichia coli* bacteriophage E3. *Kor. Jour. Microbiol.* **32**, 464-470.
5. **Kwon, O.S.,** 1994. Isolation of coliphages from soil and identification by RAPD analysis. *J. Inst. Nat. Sci.* **13**, 89-97.
6. **Kwon, Y.M., D.S. Yim, H.J. Lee, K.K. Cho, Y.J. Choi, and M.G. Baik,** 1995. Random amplified polymorphic DNA patterns within Holstein cows and among various cattle breeds. *Mol. Cells.* **5**, 393-396.
7. **Ralph, D., M. McClelland, J. Welsh, G. Baranton, and P. Perolat,** 1993. Leptospira species categorized by arbitrary primed polymerase chain reaction(PCR) and mapped restriction polymorphisms in PCR-amplified rRNA genes. *J. Bacteriol.* **175**, 973-981.
8. **Saiki, R.K.,** 1989. The design and optimization of the PCR, p.7-16. In H.A. Erlich (ed.), PCR technology: Principles and Applications for DNA Amplification. M. Stockton Press, New York.
9. **Sambrook, J., E.F. Fritsch, and T. Maniatis,** 1989. Molecular cloning: a laboratory manual, p. 9.16-9.19. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
10. **Sinsheimer, R.L.,** 1959. Purification and propertiess of bacteriophage ϕX174. *J. Mol. Biol.* **1**, 37-42.
11. **Welsh, J. and M. McClelland,** 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **18**, 7213-7219.
12. **Williams, J.G.K., M.K. Hanafey, J.A. Rafalski, and S. V. Tingey,** 1993. Genetic analysis using random amplified polymorphic DNA markers. Methods Enzymol. **218**, 704-740.
13. **Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski, and S. V. Tingey,** 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**, 6531-6535.
14. **Williams, J.G.K., A.R. Kubelik, J.A. Rafalski, and S.V. Tingey,** 1991. Genetic analysis with RAPD markers, p. 431-439. *In* J.W. Benette and L.L. Lasure (eds.), More gene manipulations in fungi, Academic press, San Diego.

15. **Williams, S., A.M. Mortimer, and J. Eccleston,** 1994. Bacteriophages in soil, p. 121-126. *In* R.G. Webster and A. Granoff (eds.), Encyclopedia of Virology. Academic Press, New York.

16. **Yoon, C.S. and K.S. Bae,** 1995. Genetic relationships among *Penicillium* species by characterizing RAPD markers. *Jour. Microbiol.* **33**, 171- 177.